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## Osteogenic protein-1 and related bone morphogenetic proteins regulate dendritic growth and the expression of microtubule-associated protein-2 in rat sympathetic neurons

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### Abstract

Osteogenic protein-1 (OP-1) is expressed in the developing nervous system and it has been found to induce dendritic growth in sympathetic neurons. To further characterize this phenomenon, the effects of OP-1 were compared to those of other members of the bone morphogenetic protein (BMP) family of growth factors. Recombinant human OP-1, BMP-6, BMP-2 and the Drosophila 60A protein induced dendritic growth in rat sympathetic neurons in a concentration-dependent manner with EC<sub>50</sub>-values of 1.8, 1.0, 1.7 and 2.7 ng/ml, respectively. In contrast, BMP-3 and cartilage-derived morphogenetic protein-2 (CDMP-2) as well as other classes of growth factors were inactive at concentrations up to 50 ng/ml. The dendritic growth induced by OP-1, BMP-6, BMP-2 and 60A was accompanied by increased expression of microtubule-associated protein-2 (MAP2) without changes in the expression of the phosphorylated forms of the M and H neurofilament subunits. These results suggest that several members of the BMP family have the capacity to regulate the morphological development of sympathetic neurons and that they may act by induction of specific cytoskeletal proteins. © 1998 Elsevier Science Ireland Ltd.

**Keywords:** Osteogenic protein-1; Bone morphogenetic protein; Dendrite; Sympathetic neurons; Microtubule-associated protein-2

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Osteogenic protein-1 (OP-1) is a member of the bone morphogenetic protein (BMP) family of growth factors. OP-1 was originally identified using a bioassay that measured bone formation [17]. However, this protein is also expressed in the developing and mature nervous system [3,4] and *in vitro* studies indicate that it can profoundly affect many aspects of neuronal development [2,12,14–16,18], including dendritic growth [10,11]. Since dendrites are the primary site of synapse formation, we felt it was important to further characterize the effects of members of the BMP family of proteins on the growth of these processes. Based on sequence similarity, BMPs can be currently divided into three subgroups [6,8,9]. The 60A subgroup consists of OP-1 (aka BMP-7), OP-2 (aka BMP-8), BMP-5, BMP-6 (aka Vgr1) and Drosophila 60A. BMP-

2, BMP-4 and Drosophila decapentaplegic (dpp) constitute the dpp subgroup. Members of the third subgroup include BMP-3, cartilage-derived morphogenetic protein-2 (CDMP-2)/BMP-13 and all other BMPs. Members of both the 60A and dpp subgroups have been found to have effects on neuronal cell development [3,7,11,12,16,19]. However, the responses of various neuronal subpopulations have been variable, with some types of cells responding to both groups of growth factors and others responding to only one group. In this study, we compared the effects of members from each of the BMP subgroups on dendritic growth. In addition, to further understand how these growth factors selectively stimulate dendritic growth, we have examined their effects on the expression of cytoskeletal proteins.

Sympathetic neurons were dissociated from the superior cervical ganglia of Holtzman (Harlan Sprague–Dawley) rat fetuses (E21) or pups (1 day postnatal) and then plated onto poly-D-lysine-coated coverslips according to the method of

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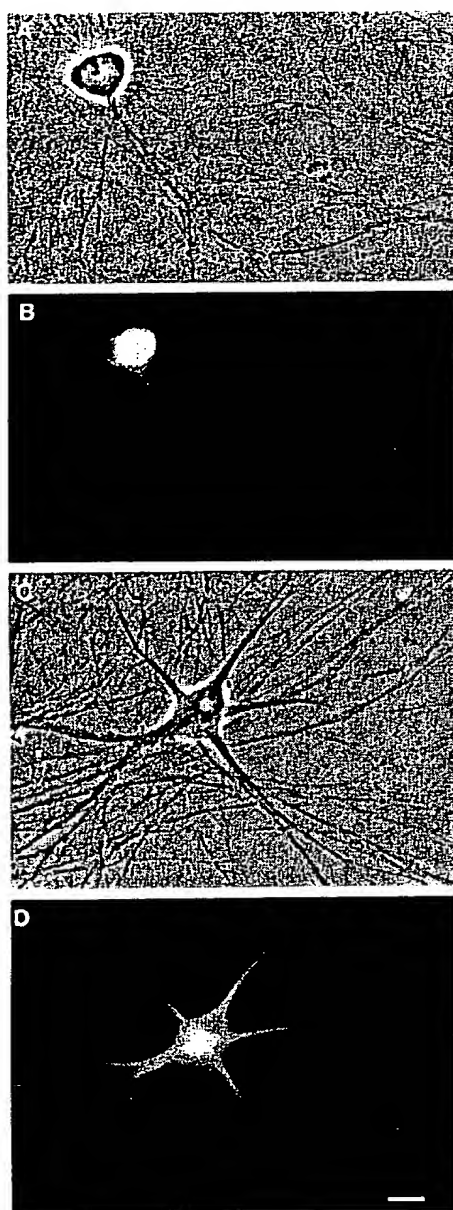


Fig. 1. Effects of BMP-6 on the morphological development of sympathetic neurons. Phase-contrast (A,C) and fluorescence (B,D) micrographs of neurons immunostained with an antibody (SMI32) to the non-phosphorylated forms of the M and H neurofilament subunits. Neurons in control cultures (A,B) typically had no dendrites whereas cells exposed to OP-1 (50 ng/ml for 5 days) (C,D) usually had several branched and tapered dendrites. Scale bar, 50  $\mu$ m.

Higgins et al. [5]. The cells were maintained in a serum-free medium that contains nerve growth factor (100 ng/ml), and non-neuronal cells were eliminated by exposure to cytosine- $\beta$ -D-arabinofuranoside (1  $\mu$ M) for 48–72 h beginning on the second day after plating. The morphology of the neurons was assessed by intracellular injection of the fluorescent dye Lucifer yellow (4%) and by immunostaining with dendrite-

specific antibodies. These included monoclonal antibody SMI32 (Sternberger Monoclonals) which recognizes n-phosphorylated epitopes on the H and M neurofilament subunits and AP20 (Sigma) and SMI52 (Sternberger Monoclonals) which both react with microtubule-associated protein-2 (MAP2). Highly purified recombinant human proteins (OP-1, BMP-2, BMP-3, BMP-6 and CDMP-2) and *Drosophila* 60A were prepared by previously published methods [17] and provided by Creative Biomolecules.

For Western blot analysis of cytoskeletal proteins, sympathetic neurons were plated onto poly-D-lysine coated 35 mm dishes and treated with 50 ng/ml of various BMPs for 5 days. Cells were then scraped off dishes in 50 mM Tris buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol and 1 mM EDTA and homogenized by passing through a 23 gauge needle at 4°C. Cell extracts were centrifuged at 12 000  $\times$  g for 15 min and the protein concentrations of the supernatants were determined using the Bradford dye reagent (Bio-Rad). Equal amounts of proteins were resolved by SDS-PAGE, electrophoretically transferred onto a nitrocellulose membrane, and probed with antibodies to MAP2 or an antibody (SMI31; Sternberger Monoclonals) to the phosphorylated forms of the H and M neurofilament subunits. Detection was accomplished using Chemiluminescent Substrate (Pierce Chemical) after sequential treatment with biotinylated goat anti-mouse IgG (HyClone) and with horseradish peroxidase-conjugated streptavidin (Amersham).

Sympathetic neurons typically extend only a single axonal process when they are grown in serum-free medium [11] and this unipolar morphology persists for several months

Table 1

Effects of different growth factors on dendritic growth in rat sympathetic neurons

Growth factor	Dendrites per cell	Cells with dendrites (%)	Length of the longest dendrite ( $\mu$ m)	EC <sub>50</sub> (ng/ml)
Control	0.2 $\pm$ 0.1	13	6 $\pm$ 4	
OP-1	4.6 $\pm$ 0.5*	100	112 $\pm$ 6*	1.8
BMP-6	5.5 $\pm$ 0.4*	100	123 $\pm$ 7*	1.0
BMP-2	4.9 $\pm$ 0.5*	100	107 $\pm$ 6*	1.7
60A	1.2 $\pm$ 0.3*	73	51 $\pm$ 9*	2.7
BMP-3	0.4 $\pm$ 0.2	25	16 $\pm$ 8	
CDMP-2	0.3 $\pm$ 0.1	18	13 $\pm$ 7	
TGF- $\beta$ 1	0.1 $\pm$ 0.1	11	7 $\pm$ 5	
GDNF	0.3 $\pm$ 0.2	20	13 $\pm$ 8	
BDNF	0.4 $\pm$ 0.2	29	19 $\pm$ 8	
NT-3	0.3 $\pm$ 0.1	18	9 $\pm$ 5	
NT-4	0.3 $\pm$ 0.2	17	7 $\pm$ 4	

Sympathetic neurons were exposed to 50 ng/ml of each of the growth factors for 5 days and then immunostained with a dendrite specific antibody (SMI32). Cellular morphology was analyzed by fluorescence microscopy using Metamorph software (Universal Imaging). Data are presented as the mean  $\pm$  SEM ( $n$  = 20–30). \* $P$  < 0.01 versus control (Student's  $t$ -test). TGF, transforming growth factor; GDNF, glia-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin.

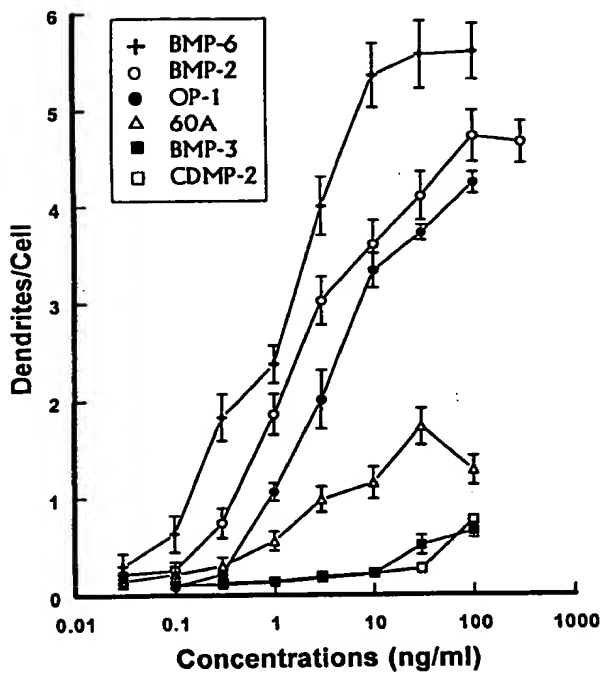


Fig. 2. Concentration-effect relationships for the effects of different BMPs on dendritic growth. Sympathetic neurons were exposed to various concentrations of BMP-6, BMP-2, OP-1, 60A, BMP-3 or CDMP-2 beginning on the 5th day in vitro and then immunostained on day 10 with an antibody (SMI32) to the non-phosphorylated forms of the M and H neurofilament subunits. Data are presented as the mean  $\pm$  SEM ( $n = 30$ ).

(Fig. 1). Treatment with maximally effective concentrations (50 ng/ml) of OP-1, BMP-6 or BMP-2 for 5 days caused virtually all of the neurons to form dendrites (Table 1). These processes exhibited a distinct taper, branched at 'Y' shaped angles, and extended  $\sim 100 \mu\text{m}$  from the cell bodies after 5 days of treatment (Fig. 1). Examination of concentration effect relationships (Fig. 2) revealed that the  $\text{EC}_{50}$ -values for BMP-6 (1.0 ng/ml) and BMP-2 (1.7 ng/ml) were similar to the  $\text{EC}_{50}$ -value for OP-1 (1.8 ng/ml) and that maximally effective concentrations of these three growth factors usually had equivalent effects on the cells as assessed by both the number of dendrites per cell and the

length of the longest dendrite (Table 1). Moreover, the effects of OP-1 and BMP-6 were not additive (data not shown) suggesting that the two ligands may share aspects of a common signaling pathway.

60A also stimulated dendritic growth and the  $\text{EC}_{50}$  (2.7 ng/ml) for this activity was similar to that for OP-1 and BMP-6. However, 60A was less efficacious and at maximally effective concentrations caused cells to form fewer dendrites (1.2/cell) than either OP-1 or BMP-6 (4.6 or 5.5/cell, respectively). At the highest concentration tested (100 ng/ml), BMP-3 and CDMP-2 produced a slight but statistically significant increase in dendritic growth. However, both BMP-3 and CDMP-2 as well as other growth factors belonging to the TGF- $\beta$  family (TGF- $\beta$ 1 and GDNF) or the neurotrophin family (BDNF, NT-3 and NT-4) were inactive at concentrations up to 50 ng/ml (Table 1). It therefore appears that dendrite promoting activity is primarily associated with the dpp and 60A subgroups of the BMP family and the structural requirements for this activity are stringent. Thus OP-1, BMP-6, BMP-2 and 60A, which share a high sequence identity (89–90%) in the conserved 7-cysteine domain of BMPs, promote dendritic growth, whereas BMP-3 and CDMP-2, which exhibit 78 and 82% identity to the 7-cysteine domain of OP-1, respectively, fail to induce dendritic growth.

High molecular weight forms of MAP2 are expressed primarily in the dendrosomatic compartment and are excluded from the axons of most mature neurons [13]. Moreover, suppression of MAP2 expression has been shown to inhibit neurite formation [1]. Therefore, we considered the possibility that BMP-induced dendritic growth might be associated with increased expression of MAP2 in cultured sympathetic neurons. Cultures exposed to OP-1, BMP-6, BMP-2 or 60A for 5 days exhibited significant increases ( $2.3 \pm 0.4$ ,  $2.9 \pm 0.5$ ,  $3.0 \pm 0.4$  or  $1.8 \pm 0.3$  fold, respectively) in the expression of high molecular weight forms of MAP2 when compared to control cultures whereas cultures exposed to CDMP-2 did not (Fig. 3). Moreover, the efficacy of the various agents in increasing MAP2 expression correlated with their ability to induce dendritic growth. In contrast, none of the agents tested affected the expression of the phosphorylated forms of the H and M neurofilament subunits which are primarily found in axons.

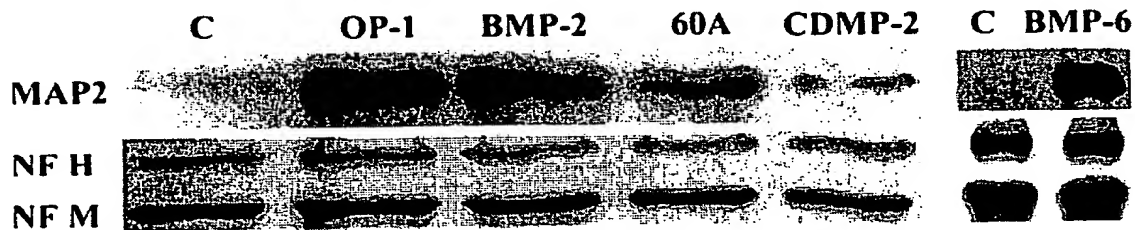


Fig. 3. Effects of various BMPs on the expression of cytoskeletal proteins. After non-neuronal cells had been eliminated, sympathetic neurons were treated with control medium (C) or with 50 ng/ml of OP-1, BMP-6, BMP-2, 60A or CDMP-2 for 5 days. Cultures were then solubilized and subjected to Western blot analysis for MAP2 which had an apparent molecular weight of 280 kDa and for phosphorylated forms of the H (NF H) and M (NF M) neurofilament subunits which had apparent molecular weights of 200 and 160 kDa, respectively. The chemiluminescent autographs are representative of three experiments which yielded similar results.

It has been reported that BMPs can modulate neural differentiation by increasing the expression of molecules involved in cell adhesion and in the synthesis of neurotransmitters [2,14–16,19]. Our data indicate that exposure to BMPs can also produce changes in the composition of the neuronal cytoskeleton and that these growth factors selectively enhance the expression of an MAP which is found in dendrites and is required for the growth of these processes. These observations suggest that regulation of MAP2 expression could be one of the mechanisms by which BMPs regulate the morphological development of sympathetic neurons.

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- [1] Caceres, A., Mautino, J. and Kosik, K.S., Suppression of MAP2 in cultured cerebellar macroneurons inhibits minor neurite formation, *Neuron*, 9 (1992) 607–618.
- [2] Fann, M.-J. and Patterson, P.H., Depolarization differentially regulates the effects of bone morphogenetic protein (BMP)-2, BMP-6, and activin A on sympathetic neuronal phenotype, *J. Neurochem.*, 63 (1994) 2074–2079.
- [3] Gross, R.E., Mehler, M.F., Mabie, P.C., Zang, Z., Santschi, L. and Kessler, J.A., Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells, *Neuron*, 17 (1996) 595–606.
- [4] Helder, M.N., Ozkaynak, E., Sampath, K.T., Luyten, F.P., Latin, V., Oppermann, H. and Vukicevic, S., Expression pattern of osteogenic protein-1 (bone morphogenetic protein-7) in human and mouse development, *J. Histochem. Cytochem.*, 43 (1995) 1035–1044.
- [5] Higgins, D., Lein, P., Osterhout, D. and Johnson, M.I., Tissue culture of autonomic neurons. In G. Banker and K. Goslin (Eds.), *Culturing Nerve Cells*, MIT Press, Cambridge, MA, 1991, pp. 177–205.
- [6] Hogan, B.L., Bone morphogenetic proteins in development, *Curr. Opin. Genet. Dev.*, 6 (1996) 432–438.
- [7] Iwasaki, S., Hattori, A., Sato, M., Tsujimoto, M. and Kohno, M., Characterization of the bone morphogenetic protein-2 as a neurotrophic factor. Induction of neuronal differentiation of PC12 cells in the absence of mitogen-activated protein kinase activation, *J. Biol. Chem.*, 271 (1996) 17360–17365.
- [8] Kingsley, D.M., The TGF- $\beta$  superfamily: new members, new receptors, and new genetic tests of function in different organisms, *Genes Dev.*, 8 (1994) 133–146.
- [9] Kriegstein, K., Rufer, M., Suter-Crazzolara, C. and Unsicker, K., Neural functions of the transforming growth factors  $\beta$ , *Int. J. Dev. Neurosci.*, 13 (1995) 301–315.
- [10] Lein, P., Guo, X., Hedges, A.M., Rueger, D., Johnson, M. and Higgins, D., The effects of extracellular matrix and osteogenic protein-1 on the morphological differentiation of rat sympathetic neurons, *Int. J. Dev. Neurosci.*, 14 (1996) 203–215.
- [11] Lein, P., Johnson, M., Guo, X., Rueger, D. and Higgins, D., Osteogenic protein-1 induces dendritic growth in rat sympathetic neurons, *Neuron*, 15 (1995) 597–605.
- [12] Liem, K.F. Jr., Tremml, G., Roelink, H. and Jessell, T.M., Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm, *Cell*, 82 (1995) 969–979.
- [13] Matus, A., Stiff microtubules and neuronal morphology, *Trends Neurosci.*, 17 (1994) 19–22.
- [14] Perides, G., Hu, G., Rueger, D.C. and Charness, M.E., Osteogenic protein-1 regulates L1 and neural cell adhesion molecule gene expression in neural cells, *J. Biol. Chem.*, 268 (1993) 25197–25205.
- [15] Perides, G., Safran, R.M., Downing, L.A. and Charness, M.E., Regulation of Neural cell adhesion molecule and L1 by the transforming growth factor- $\beta$  superfamily. Selective effects of the bone morphogenetic proteins, *J. Biol. Chem.*, 269 (1994) 765–770.
- [16] Reissmann, E., Ernsberger, U., Francis-West, P.H., Rueger, D., Brickell, P.M. and Rohrer, H., Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons, *Development*, 122 (1996) 2079–2088.
- [17] Sampath, T.K., Maliakal, J.C., Hauschka, P.V., Jones, W.K., Sasak, H., Tucker, R.K., White, K.H., Coughlin, J.E., Tucker, M.M., Pang, R.H.L., Corbett, C., Ozkaynak, E., Oppermann, H. and Rueger, D.C., Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with mature bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro, *J. Biol. Chem.*, 267 (1992) 20352–20362.
- [18] Shah, N.M., Groves, A.K. and Anderson, D.J., Alternative neural crest cell fates are instructively promoted by TGF $\beta$  superfamily members, *Cell*, 85 (1996) 331–343.
- [19] Varley, J.E. and Maxwell, G.D., BMP-2 and BMP-4, but not BMP-6, increase the number of adrenergic cells which develop in quail trunk neural crest cultures, *Exp. Neurol.*, 140 (1996) 84–94.